

Molecular profiling of allergen-specific antibody responses may enhance success of specific immunotherapy



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Background: House dust mites (HDMs) are among the most important allergen sources containing many different allergenic molecules. Analysis of patients from a double-blind, placebo-controlled allergen-specific immunotherapy (AIT) study indicated that patients may benefit from AIT to different extents depending on their molecular sensitization profiles.

Objective: Our aim was to investigate in a real-life setting whether stratification of patients with HDM allergy according to molecular analysis may enhance AIT success.

Methods: Serum and nasal secretion samples from patients with HDM allergy (n = 24) (at baseline, 7, 15, 33, and 52 weeks) who had received 1 year of treatment with a well-defined subcutaneous AIT form (Alutard SQ 510) were tested for IgE and IgG reactivity to 15 microarrayed HDM allergen molecules with ImmunoCAP Immuno-solid-phase Allergen Chip technology. IgG subclass levels to allergens and peptides were determined by ELISA, and IgG blocking was assessed by

basophil activation. *In vitro* parameters were related to reduction of symptoms determined by combined symptom medication score and visual analog scale score.

Results: Alutard SQ 510 induced protective IgG mainly against *Dermatophagoides pteronyssinus* (Der p) 1 and Der p 2 and to a lesser extent to Der p 23, but not to the other important allergens such as Der p 5, Der p 7, and Der p 21, showing better clinical efficacy in patients sensitized only to Der p 1 and/or Der p 2 as compared with patients having additional IgE specificities.

Conclusion: Stratification of patients with HDM allergy according to molecular sensitization profiles and molecular monitoring of AIT-induced IgG responses may enhance the success of AIT. (J Allergy Clin Immunol 2020;146:1097-108.)

Key words: House dust mite, immunotherapy, recombinant allergens, basophil activation, IgE, IgG, IgG₁, IgG₂, IgG₃, IgG₄, IgG subclass, allergen microarray

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Allergen-specific immunotherapy (AIT) is the only disease-modifying treatment for patients with allergy that has long-lasting effects.^{1,2} AIT seems to be even more effective than pharmacotherapy in reducing symptoms,^{3,4} and it prevents the progression from mild forms of allergy (rhinoconjunctivitis) to severe forms (asthma).⁵ However, several factors, such as long duration of treatment with multiple applications and potential side effects, lead to poor compliance of patients and thus limit utilization of AIT.^{6,7} Another important problem relates to difficulties in obtaining high-quality allergen preparations for the formulation of AIT vaccines.⁸ The latter problem is particularly relevant for house dust mites (HDMs), which contain a variety of different allergen molecules.^{9,10} HDMs are among the most important allergen sources worldwide, and they are responsible for severe forms of respiratory and cutaneous allergic manifestations.¹¹ Because HDMs are rich in proteases and immunomodulatory components,¹² it is difficult to obtain high-quality allergen extracts. In the HDM, *Dermatophagoides pteronyssinus* (Der p) 1, Der p 2, Der p 5, Der p 7, Der p 21, and Der p 23 seem to represent the most clinically relevant allergens^{13,14}; however, most allergen extracts prepared from natural allergen sources contain mainly Der p 1 and Der p 2, whereas the other allergens occur in small amounts or are missing. In this context, it has been demonstrated that important allergens were missing from HDM allergen extracts used for skin testing.¹⁵

Although several studies have demonstrated clinical efficacy of subcutaneous AIT (SCIT) for the treatment of HDM allergy¹⁶⁻¹⁸ it

Abbreviations used

AIT:	Allergen-specific immunotherapy
CSMS:	Combined symptom and medication score
Der f:	<i>Dermatophagoides farinae</i>
Der p:	<i>Dermatophagoides pteronyssinus</i>
HDM:	House dust mite
ISAC:	Immuno-solid-phase Allergen Chip
Phl p:	<i>Phleum pratense</i>
rDer p:	Recombinant <i>Dermatophagoides pteronyssinus</i>
SCIT:	Subcutaneous immunotherapy
SPT:	Skin prick test
VAS:	Visual analog scale

seems that SCIT for HDM allergy is less effective than SCIT for seasonal allergies such as grass pollen allergy when vaccines of the same type are compared (ie, when SCIT is compared with the aluminium hydroxide-adsorbed allergen extract Alutard).¹⁹⁻²² In fact, SCIT with the aluminium hydroxide-adsorbed HDM allergen extract Alutard has been studied quite extensively in clinical studies in adults and children.^{23,24} Analysis of the underlying immunologic mechanisms indicated that the induction of allergen-specific IgG₄ antibodies not only served as a marker for the immunologic response but was also partly associated with clinical efficacy.²³ Accordingly, the induction of allergen-specific IgG, and the induction of allergen-specific IgG₄ in particular, is currently considered an important biomarker for effects and, in part, efficacy of AIT.^{25,26} Further support for the importance of allergen-specific IgG antibodies in reducing allergic symptoms comes from a recent study demonstrating that passive immunization with allergen-specific IgG mAbs potently suppressed allergic symptoms in a clinical study.²⁷ However, analysis of the induction of allergen-specific IgG₄ and the IgE-blocking activity with crude HDM allergen extracts showed modest correlations between clinical efficacy and IgE-blocking activity of the therapy-induced IgG₄ response.²³ Because HDMs (unlike cat, birch pollen, or grass pollen) contain several different important allergens that may not be well represented in natural allergen extracts, it is tempting to speculate that patients with different profiles of IgE reactivity to HDM allergens may respond in a different manner depending on presence of the individual HDM allergens in the vaccine. In fact, the *post hoc* analysis of IgE and IgG responses of sera from patients undergoing a clinical trial with HDM SCIT in which clinical effects were recorded in an allergen challenge chamber indicated that the reduction of total nasal symptom scores may be related to the patients' IgE recognition profiles and development of allergen-specific IgG.²⁸

Here we have performed the first real-life study using a large panel of purified HDM allergen molecules and analyzed the molecular IgE sensitization profiles and development of allergen-specific IgG antibodies in patients with HDM allergy who were receiving SCIT with Alutard and in a parallel control group without SCIT. In particular, we performed a detailed analysis of allergen-specific IgG subclass responses. The ability of treatment-induced IgG to inhibit allergen-induced basophil degranulation was studied with multiple allergens, and immunologic parameters were related to clinical outcome. The results of our study indicate that molecular diagnosis and molecular monitoring of AIT-induced IgG antibodies may be useful to predict and monitor efficacy of HDM AIT in real life.

METHODS**Subjects and treatment**

We analyzed serum samples from 24 patients with HDM allergy who had been treated with conventional HDM SCIT (Alutard-SQ 510, ALK-Abelló, The Netherlands) (a group of patients with HDM allergy who received immunotherapy [referred to as the HDMIT group]), a group of 9 patients with HDM allergy without immunotherapy (referred to as the HDMA group), and a group of nonallergic subjects.²⁹ Patients with HDM allergy had a positive skin prick test (SPT) result with HDM, a level of Der p-specific IgE antibodies greater than 0.35 kUA/L determined by ImmunoCAP (Phadia AB/ThermoFisher, Uppsala, Sweden), and a clinical history of symptoms of allergy to HDM. AIT was conducted for a 3-year period, and monitoring and sample collection were performed for the first 12 months. One group of patients with allergy (the HDMIT group) received weekly subcutaneous injections for 15 weeks, with monthly injections for 1 year afterward. The dosage was an escalation regimen from 100, 1,000, and 10,000 to 100,000 standardized quality units per milliliter, which was reached at the end of the initiation (15 weeks) and maintained for the remaining year (Fig 1). All patients were treated at the Ghent University Hospital, in Ghent, Belgium; all data and samples were collected prospectively. At each visit—at baseline (at visit 0 [V0], halfway through initiation (7 weeks [V1]), at the end of initiation (15 weeks [V2]), after 8 months of maintenance (V3), and after 1 year of treatment (V4) (Fig 1)—patients filled out questionnaires, underwent SPTs, had their clinical symptoms assessed (with use of visual analog scale [VAS] scores and combined symptom medication scores [CSMSs]), and had blood and nasal secretions collected as described.³⁰ During the same period, serum samples and nasal secretions were obtained from the parallel HDMA group (n = 9) and from the group of nonallergic subjects (n = 9). The study was approved by the local ethical committee of Ghent University Hospital (EC 2013/672). Written informed consent was obtained from each patient, including permission to investigate their blood samples. Patients' baseline characteristics and immunologic parameters are displayed in Table I. The anonymized serum samples were analyzed with permission from the ethics committee of the Medical University of Vienna (EK 1641/2014).

ImmunoCAP ISAC assay

Serum samples from the HDMIT group (n = 24), the HDMA group (n = 9), and the group of nonallergic subjects (n = 9) that had been collected at every visit (V0-V4) were analyzed for IgE and IgG reactivity to multiple microarrayed allergen molecules, including 15 HDM allergens (ie, Der p 1, *Dermatophagoides farinae* 1 [Der f 1], Der p 2, Der f 2, Der p 4, Der p 5, Der p 7, Der p 10, Der p 11, Der p 14, Der p 15, Der p 18, Der p 21, Der p 23, and Der p 37). The samples were analyzed by using the MeDALL chip, which is based on Immuno-solid-phase Allergen Chip (ISAC) microarray technology (Phadia Multiplexing; ThermoFisher Scientific) as described.³¹ For IgE measurement, serum samples were used undiluted, whereas for IgG measurement, sera were diluted 1:50. Results were evaluated by using Phadia Microarray Image Analysis software, and levels of allergen-specific IgE and IgG antibodies were reported in ISAC standardized quality units for IgE antibodies or ISAC standardized quality units for IgG antibodies, with a cutoff of 0.3 ISAC standardized quality units.

HDM allergens and peptides

Natural purified Der p 1 was obtained from Professor W. R. Thomas.³² Recombinant Der p (rDer p) 2, rDer p 23, rDer p 5, and rDer p 21 were expressed in *Escherichia coli* and purified as described.³³⁻³⁵ The expression and purification of rDer p 7 are described in the Online Repository.

Peptides of Der p 1, Der p 2, and Der p 23 with a length between 29 and 43 amino acids, covering the sequences of the allergens (see Table E1 in this article's Online Repository at www.jacionline.org), were synthesized by using a 9-fluorenylmethoxycarbonyl strategy as described elsewhere.³⁶ The peptides were identified by mass spectrometry and purified by preparative HPLC to a purity greater than 90%.³⁷ The sequences and numbers of amino acids of each of the peptides, their location in the corresponding allergen, and the ability of peptide-specific IgG antibodies to inhibit IgE binding in patients with allergy, as determined in earlier studies, are shown in Table E1.

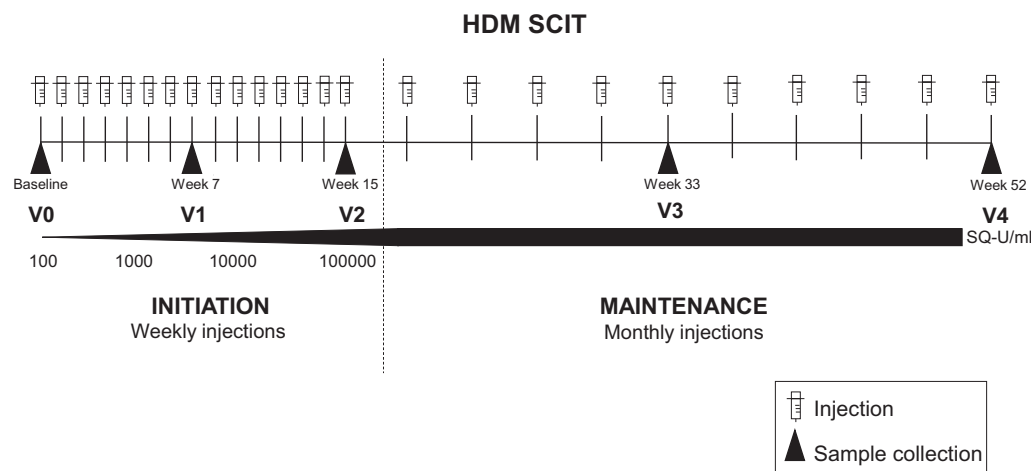


FIG 1. Scheme of AIT treatment and sample collection. Patients received subcutaneous AIT consisting of a 15-week up dosing period with 15 weekly injections (initiation) and monthly maintenance injections for 1 year (maintenance). Blood and nasal secretions were collected at visits 0 to 4, as indicated. *SQ-U*, Standardized quality units.

TABLE I. Demographic and clinical characteristics of subjects included in the study

Group	HDMIT group	HDMA group	Patients without allergy
No.	24	9	9
Median age, y	31.44	27.73	26.87
Sex (male/female)	9/15	4/5	3/6
Median length (cm)	170	170	168
Median weight (kg)	63.5	73	64
Median BMI (kg/m ²)	21.65	22.84	23.71
Asthma (%)	46.67	11.11	na
Rhinitis (%)	100	100	na
Median total IgE, kU/L (range)	134.74 (16.45-1975.84)	80.06 (27.67-374.30)	13.81 (3.15-107.89)
Median <i>D pteronyssinus</i> sIgE level, kUA/L (range)	0.05-200.91 (19.43)	0.39-69.37 (4.87)	na
Median <i>D farinae</i> sIgE level, kUA/L (range)	0.05-188.20 (12.78)	0.21-49.61 (5.35)	na
Monosensitized to HDM, n (%)	6 (25.00)	4 (44.44)	na
Cosensitizations, n (%)	18 (75.00)	5 (55.55)	na
Grass, n (%)	16 (66.66)	4 (44.44)	na
Tree, n (%)	9 (37.50)	1 (11.11)	na
Weed, n (%)	3 (12.50)	na	na
Mold, n (%)	4 (16.66)	1 (11.11)	na
Cat/dog, n (%)	15 (62.50)	2 (33.33)	na

HDMA, Patients with HDM allergy who did not receive immunotherapy; HDMIT, patients with HDM allergy who received immunotherapy; na, not applicable.

ELISA assays

Allergen- and peptide-specific human IgG₁, IgG₂, IgG₃, and IgG₄ subclass responses were measured by ELISA.^{38,39} For this purpose, serum samples were diluted 1:50 and nasal secretions were diluted 1:4 in PBS containing 0.5% Tween 20. OD values were recorded on a FLUOstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany). All determinations were performed in duplicate, and the results were displayed as means of the OD values with deviations less than 5%. OD values from different plates were harmonized by including standards on each plate.

Quantitative IgG₁ and IgG₄ ELISA measurements were performed by using 96-well ELISA plates (Nunc Maxisorp, Roskilde, Denmark). Each well was coated with 100 μ L of natural Der p 1, rDer p 2, or rDer p 23 (2 μ g/mL in 100 mM carbonate buffer [pH 9.6]), after which the plates were incubated for 5 hours at room temperature. After being washed with 0.05% vol/vol Tween-20 (Sigma-Aldrich, St Louis, Mo) in PBS and being blocked with 2% wt/vol BSA in PBS/0.05% Tween 20 overnight, the plates were incubated with patients' sera (diluted 1:50 and 1:100 in PBS/0.05% Tween-20/0.5% BSA) for 2 hours at 37°C and 1 hour at room

temperature. Standard curves for IgG₁ and IgG₄ were established by coating plates with 2 μ g/mL of *Phleum pratense* (Phl p) 2 and Phl p 5 and incubating them with a human IgG₁ mAb specific for Phl p 2⁴⁰ or with a human IgG₄ mAb specific for Phl p 5⁴¹ with mAb concentrations of 50, 150, 450, 900, and 1350 ng/mL diluted in PBS/0.05% Tween-20/2% BSA in triplicate for 2 hours at 37°C and 1 hour at room temperature. Bound antibodies were detected with biotinylated subclass-specific antibodies (mouse anti-human IgG₁ mAb (clone G17-1) or IgG₄ (clone G17-4) (BD, San Diego, Calif) (diluted 1:1000 in PBS/0.05% Tween-20/0.5% BSA) mixed with horseradish peroxidase-conjugated streptavidin (BD Pharmingen, catalog no. 554066) (diluted 1:2500 in PBS/0.05% Tween-20/0.5% BSA) overnight at 4°C. The substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma-Aldrich) was added at a rate of 100 μ L per well, and the ODs were measured by using an ELISA Reader (Tecan Infinite F50, Männedorf, Switzerland) at 405/490 nm. Concentrations of allergen-specific IgG₁ and IgG₄ were calculated according to the standard curve of OD values obtained for known concentrations.

Basophil activation and inhibition of basophil activation with blocking IgG

Rat basophilic leukemia RBL cells (clone RS-ATL8)⁴² transfected with the cDNA coding for the human high-affinity IgE receptor were maintained in minimum essential medium (MEM) with Earle salt supplemented with 10% FBS, 2mM L-glutamine (ThermoFisher Scientific), 100 U/mL of penicillin, and 100 µg/mL of streptomycin, 200 µg/mL of Geneticin (ThermoFisher Scientific), and 200 µg/mL of Hygromycin B (ThermoFisher Scientific) at 37°C. Cells were plated in 96-well sterile tissue culture plates (50 µL per well) with the sera (1:20 dilution in supplemented MEM) obtained from the treated patients (n = 9) before immunotherapy at baseline (ie, at visit V0) and incubated overnight at 37°C and 7% CO₂. For control purposes, cells were incubated only with supplemented MEM as a negative control. The next day, serum samples that had been obtained from the same patients before and after AIT were incubated for 1.5 hours at 56°C to inactivate IgE antibodies. Thereafter, IgE-inactivated sera diluted 1:20 in Tyrode buffer (ie, Tyrode salts dissolved in water according to the manufacturer (Sigma Aldrich), 0.02M NaHCO₃, 1% wt/vol BSA, and 50% vol/vol D₂O [7.2]) were incubated with different concentrations of purified allergens (1, 10, and 100 ng/mL) for 2 hours at 37°C and added to the cells, which had been washed 3 times with washing buffer (Tyrode salts, 0.02M NaHCO₃, and 1% wt/vol BSA in H₂O [pH 7.2]) for 1 hour at 37°C. The release of β-hexosaminidase in the medium from activated RBL cells was determined by a fluorometric assay by using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as a substrate (0.1 mM in 100 mM citrate [pH 4.5]). The reaction was stopped with 0.25 M glycine buffer after 60 minutes of incubation at room temperature. The plate was read on an Infinite M200 PRO series plate reader (TECAN Austria GmbH, Grödig, Austria) by using 380-nm excitation and 440-nm emission filters. All measurements were performed in triplicate, and the results were displayed as percentages of total release (means ± SDs).⁴³

For measuring direct mediator release, cells were loaded with serum obtained from patients before immunotherapy at baseline (V0) or afterward (V4) and exposed to different concentrations of purified allergens (0.01 to 10 ng/mL). β-Hexosaminidase release was determined as described in the preceding paragraph.⁴³

Statistics

The Mann-Whitney and Wilcoxon *U* tests were used to compare independent samples from different subjects to compare the IgE and IgG levels, as well as the IgG subclasses and allergen peptide ODs in different groups at the given different time points (GraphPad Prism software, version 7.00 for Windows, GraphPad Software, La Jolla, Calif). A *P* value less than .05 was considered statistically significant, as indicated in the figure legends.

RESULTS

Real-life study design and characterization of patients with HDM allergy

To study immune responses in patients with HDM allergy who were undergoing subcutaneous immunotherapy with HDM allergen extract-based Alutard SQ 510, an investigator-driven study was conducted at the Department of Otorhinolaryngology in Ghent University Hospital in Ghent, Belgium.²⁹ In this observational study patients with HDM allergy received subcutaneous immunotherapy with HDM allergen extract-based Alutard SQ 510 for 3 years (those in the HDMIT group); a control group without AIT (ie, those in the HDMA group) was also included. Serum samples from 24 of the 28 patients in the HDMIT group and all 9 patients in the HDMA group that had been obtained at baseline (ie, visit 0), after week 7 (ie, visit 1), after week 15 (ie, visit 2), after week 33 (ie, visit 3), and after week 52 (ie, visit 4) (Fig 1) were available for analysis. Table I provides an overview of the demographic and clinical characterization of the

HDMIT group and HDMA group. Pseudonymized serum samples and nasal secretion samples were analyzed by an investigator who was blinded regarding the 2 groups.

Table II shows the molecular IgE recognition profiles of the patients in the HDMIT group and the patients in the HDMA group when tested with 13 microarrayed *D pteronyssinus* allergen molecules, as well as with Der f 1 and Der f 2 at the baseline visit. In the HDMIT group, Der p 2 (91.7%), Der p 1 (58.3%), and Der p 23 (45.8%) were the most frequently recognized allergen molecules, followed by Der p 7 (41.7%), Der p 4 (33.3%), Der p 21 (29.2%), Der p 10 (20.9%), Der p 5 and Der p 37 (both 12.5%), Der p 18 (8.3%), and Der p 15 (4.2%). In the HDMA group, frequencies of recognition of IgE to the individual allergen molecules were lower, which was in agreement with the lower levels of Der p- and Der f-specific IgE (Table I). Fewer members of the HDMA group had asthma than in the HDMIT group (ie, 11.1% vs 46.7%). A detailed demographic, clinical, and serologic characterization of each of those in the HDMIT group and those in the HDMA group can be found in Tables E2 and E3 (in this article's Online Repository at www.jacionline.org). Patients who were sensitized only to Der p 1 and/or Der p 2 are indicated in blue and those who are sensitized against at least 1 of the additional clinically relevant allergens (ie, Der p 5, Der p 7, Der p 21, and Der p 23) are highlighted in yellow in Tables E2 and E3.

Vaccination with Alutard SQ 510 induces specific IgG responses only to Der p 1, Der p 2, and Der p 23 and not to any of the other Der p allergen molecules

Fig 2 shows the development of allergen-specific IgG levels in the HDMIT group compared with the levels in the HDMA group during the observation period of 1 year. The patients in the HDMIT group showed a significant increase in Der p 1-, Der f 1-, Der p 2-, and Der f 2-specific IgG responses in serum samples obtained at visits 2, 3, and 4 compared with baseline. A much lower but significant increase in Der p 23-specific IgG levels was noted as well for the HDMIT group (Fig 2, B). However, no relevant induction of IgG antibodies specific for any of the other HDM allergens (Der p 5, Der p 7, or Der p 21 [Fig 2, A and B] and Der p 4, Der p 10, Der p 11, Der p 14, Der p 15, Der p 18, or Der p 37 [see Fig E1, A and B in this article's Online Repository at www.jacionline.org and see also Table E3]) was found. No relevant HDM allergen-specific IgG increases were found in the untreated HDMA group (Fig 2, A and B [see Table E3]).

The analysis of allergen-specific IgE responses in the course of AIT showed that the patients in the HDMIT group also had increased levels of IgE to Der p 2, Der f 2, Der p 1, Der f 1, and Der p 23 beginning with visit 2, with the levels going down at visits 3 and 4 whereas levels of allergen-specific IgG continued to increase (Fig 2, A and B). These increases in allergen-specific IgE levels were observed only for those allergens against which IgG was induced but not for the other HDM allergen molecules. For certain patients in the HDMIT group (ie, patients 008, 030, 032, 034, 037, 039, and 040), we noted a *de novo* induction of allergen-specific IgE against allergens that were not recognized at baseline (see Table E4 in this article's Online Repository at www.jacionline.org). When basophils were loaded with sera obtained from the latter patients at baseline and after treatment we found that

TABLE II. Prevalence of IgE reactivity and mean IgE levels (ISU) to 15 HDM allergens

Group	HDMIT		HDMA	
	24		9	
No.	Prevalence (%)	Mean (SD)	Prevalence (%)	Mean (SD)
Der f 1	45.83	7.49 (12.15)	44.44	3.09 (4.45)
Der f 2	83.33	26.20 (37.43)	77.78	11.55 (23.64)
Der p 1	58.33	16.04 (27.63)	55.56	2.62 (4.79)
Der p 2	91.67	19.98 (31.83)	55.56	9.07 (18.07)
Der p 4	33.33	1.05 (0.68)	11.11	0.13 (0.22)
Der p 5	12.50	0.54 (0.16)	0.00	0.00 (0.00)
Der p 7	41.67	3.60 (3.96)	11.11	0.13 (0.34)
Der p 10	20.83	0.51 (0.17)	0.00	0.02 (0.03)
Der p 11	0.00	0.00 (0.00)	0.00	0.00 (0.00)
Der p 14	0.00	0.00 (0.00)	0.00	0.00 (0.01)
Der p 15	4.17	0.00 (0.00)	11.11	0.16 (0.47)
Der p 18	8.33	14.16 (19.37)	11.11	0.08 (0.23)
Der p 21	29.17	3.64 (5.52)	0.00	0.00 (0.00)
Der p 23	45.83	15.09 (34.66)	33.33	2.47 (7.07)
Der p 37	12.50	2.22 (2.58)	0.00	0.01 (0.03)

HDMA, Patients with HDM allergy who did not receive immunotherapy; HDMIT, patients with HCM allergy who received immunotherapy.

treatment-induced IgE led to an allergen-specific induction of mediator release in more than half of them (see Fig E2 in this article's Online Repository at www.jacionline.org). No increases in allergen-specific IgE levels were found for the untreated HDMA group (Fig 2, A and B).

Maintenance treatment continuously increases allergen-specific IgG₄ responses but only maintains IgG₁ and IgG₂ responses

Fig 3 presents the results obtained when allergen-specific IgG subclass responses were analyzed in the HDMIT group during the 1-year period of investigation. The IgG subclass analysis was performed only for those allergens against which increases in allergen-specific IgG responses were found (ie, Der p 1, Der p 2, and Der p 23) and for control purposes, for 1 allergen for which no IgG response was found (ie, Der p 5). Allergen-specific IgG subclass responses were observed mainly for IgG₁, IgG₄, and for IgG₂ but not for IgG₃, and they were limited to Der p 1, Der p 2, and Der p 23, whereas no Der p 5-specific IgG subclass responses were found (Fig 3). We noted interesting differences regarding the kinetics of the IgG subclass responses in the course of treatment: allergen-specific IgG₄ responses continued to increase during the course of treatment, as shown for Der p 1, Der p 2, and Der p 23. By contrast, allergen-specific IgG₁ and IgG₂ responses were lower and did not further increase during the maintenance treatment. Using a quantitative ELISA for measuring the concentrations of allergen-specific IgG₁ and IgG₄, we found that AIT-induced allergen-specific IgG₁ concentrations were in the range of 5 µg/mL, whereas allergen-specific IgG₄ concentrations went up to approximately 25 µg/mL (see Fig E3, A and B in this article's Online Repository at www.jacionline.org).

Allergen-specific IgG₁ and IgG₄ antibodies could be also detected in nasal secretions, but their levels were low and increases were noted only for specific IgG₄ antibodies (see Fig E4 in this article's Online Repository at www.jacionline.org).

In the untreated (HDMA) group, allergen-specific IgG subclass levels did not change during the year of investigation (data not shown).

IgG₄ antibodies are only partly directed against the IgE-binding regions of the allergens

IgG₄ levels specific for 8 overlapping peptides of Der p 1, 5 Der p 2 peptides, and 5 Der p 23 peptides, which covered the sequences of the allergens (see Table E1), were measured in sera of patients from the HDMIT group that had been obtained before immunotherapy (at V0), after 33 weeks (at V3), and after 1 year (at V4) of immunotherapy. Table E1 indicates the percentages in patients with allergy of inhibition of IgE binding to the corresponding allergens obtained with peptide-specific antibodies and thus define whether a peptide was derived from an IgE-binding region.^{44,45} Statistically significant increases in IgG₄ levels were found for all but 1 of the Der p 1 peptides (ie, peptide 1), which were associated with the most significant increases in IgG₄ against peptides 6, 7, and 8 (Fig 4). Thus, no induction of IgG₄ responses against peptide 1, which is part of the Der p 1 IgE-binding region, was found. Der p 2-specific IgG₄ antibodies induced during immunotherapy were directed against peptides 2 and 3 and most significantly against peptides 4 and 5 (Fig 4). No development of IgG₄ responses against peptide 1 from Der p 2, which is part of the IgE epitope-containing region, was found. Der p 23-specific IgG₄ antibodies recognized mainly peptide 3, which represents an IgE-reactive part of the allergen⁴⁴ (Fig 4). We therefore found that AIT-induced IgG₄ was directed against some but not all IgE-binding regions of the allergens and also reacted with areas not relevant for IgE binding.

AIT-induced IgG antibodies inhibit mainly Der p 1-, Der p 2-, and Der p 23-induced basophil activation

Next, we investigated whether AIT-induced IgG antibodies can inhibit allergen-induced basophil activation (Fig 5 and see Fig E5 in this article's Online Repository at www.jacionline.org). Examples are shown for 9 patients from the treatment group (HDMIT group), which showed IgE reactivity not only to the major HDM allergens, Der p 1, Der p 2, and Der p 23, but also to 1 or more of the other HDM allergens (Fig 5 and see Fig E5). The detailed analysis showed that an inhibition of basophil activation by post-AIT sera was usually associated with the induction of allergen-specific IgG antibodies (Fig 5 and see Fig E5).

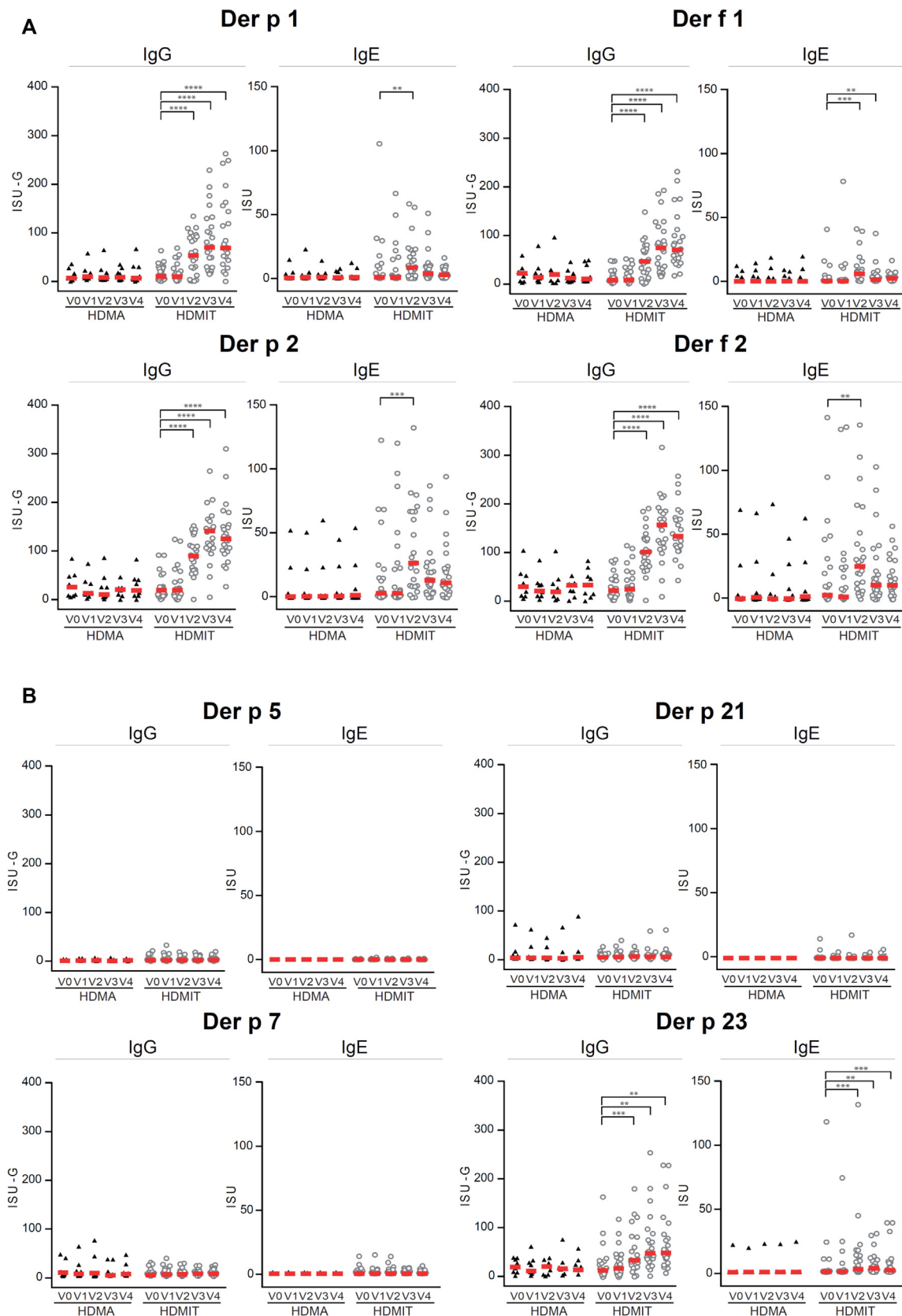


FIG 2. IgE and IgG reactivity to HDM allergen molecules before and during AIT. IgE and IgG levels (y axes show IgE in terms of standardized quality unit [ISU] and ISAC standardized quality unit for IgG antibodies [ISU-G]) to Der p 1, Der p 2, Der f 1, and Der f 2 (**A**) and Der p 5, Der p 7, Der p 21, and Der p 23 (**B**), as measured in sera of those patients in the HDMIT group (gray dots [$n = 24$]) and in the sera of those patients in the HDMA group (black triangles [$n = 9$]), as determined by ISAC at different visits (x axes show values at V0 [baseline], V1 [7 weeks], V2 [15 weeks], V3 [33 weeks], and V4 [1 year]). Red horizontal bars indicate median values. Statistically significant differences are indicated as follows: * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

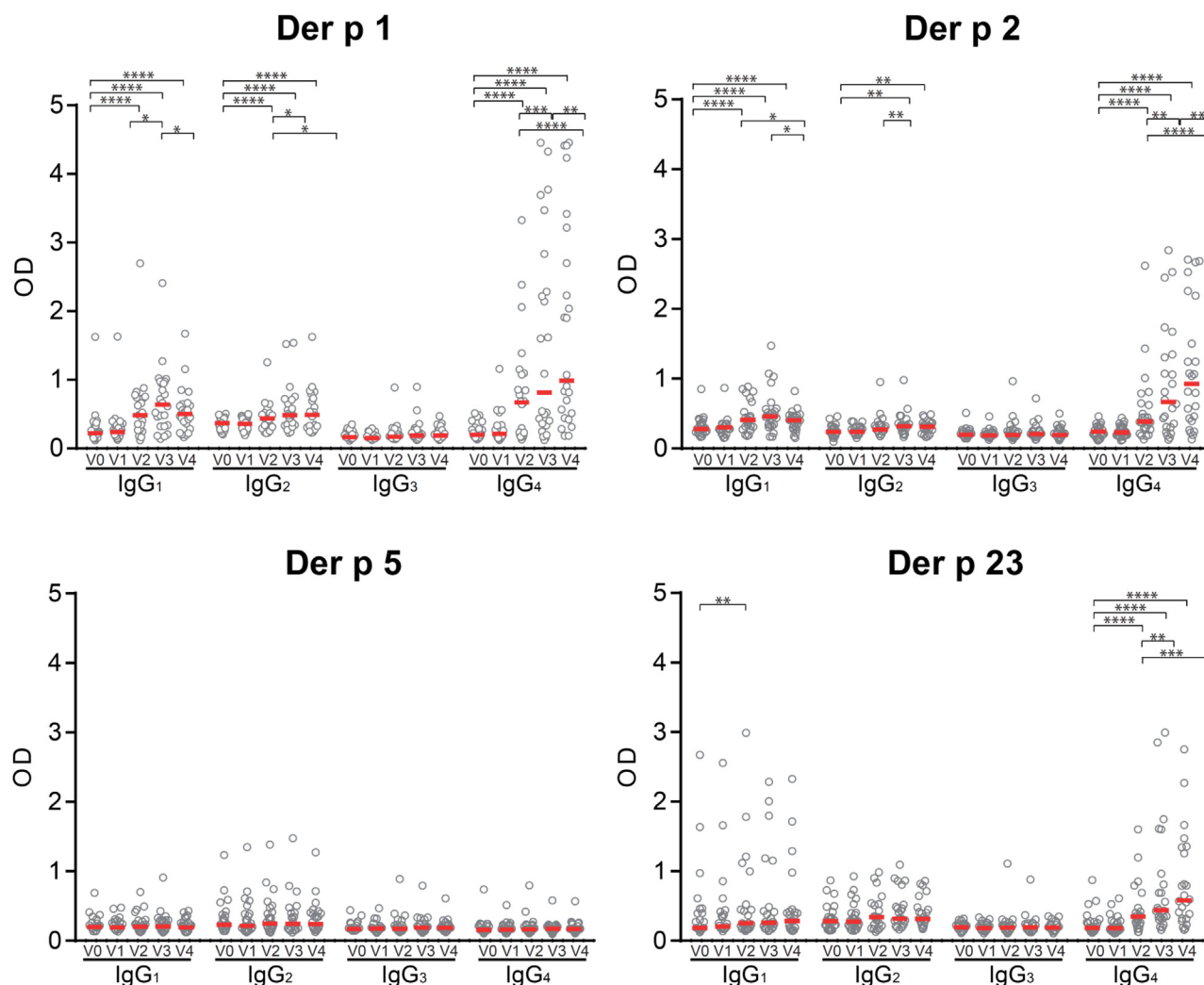


FIG 3. Development of allergen-specific IgG subclass responses in sera from patients in the HDMIT group during AIT. IgG₁, IgG₂, IgG₃, and IgG₄ levels to Der p 1, Der p 2, Der p 5, and Der p 23 (y axes show OD values) at different visits (x axes show values at V0 [baseline], V1 [7 weeks], V2 [15 weeks], V3 [33 weeks], and V4 [1 year]). Red horizontal bars indicate median values. Statistically significant differences are indicated as follows: **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

Accordingly, the post-AIT sera inhibited mainly Der p 1– and Der p 2–induced basophil activation (Fig 5, A and B and see Fig E5, A–G). In the presence of AIT-induced IgG antibodies (V4) an approximately 10-fold concentration of allergen was necessary to induce basophil degranulation as compared with the concentration in serum obtained at baseline (at V0) (Fig 5 and see Fig E5, A–G). An inhibition of Der p 23–induced basophil activation was noted only for certain Der p 23–sensitized subjects (Fig 5, B and see Fig E5, A, C, and E) but not for others (Fig 5, A and see Fig E5, B, D, F, and G). Inhibition of basophil activation was observed for Der p 7 in only 2 subjects (see Fig E5, A and B) but not for the other allergens (ie, Der p 5 and Der p 21) and not for other subjects. Der p 4 showed no or only very low allergenic activity when basophil activation was tested in sensitized patients (patients 009, 019, 021, 027, 029, 033, 037, and 038 [see Table E2 and Fig E5, H]).

Evidence that AIT with Alutard SQ 510 is clinically less effective in patients with IgE reactivity to Der p 5, Der p 7, Der p 21, and/or Der p 23

CSMSs and VAS scores were assessed for each patient at each visit (V0–V4), and then the percentages of reduction of CSMSs and VAS scores between the baseline (V0) and the last visit (at 1 year [V4]) were calculated for each of the patients in the HDMIT group. Patients were then divided in groups with a reduction of more or less than 50% in their CSMS (Fig 6 [upper part]) or in groups with a reduction of more or less than 50% in their VAS score (Fig 6 [lower part]). We found that among patients with either a CSMS (*n* = 16) or a VAS score reduction less than 50% (*n* = 14) the frequencies of IgE binding to Der p 5, Der p 7, Der p 21, and Der p 23 were higher than in patients with a CSMS (*n* = 5) or VAS score reduction greater than 50% (*n* = 10), although no statistically

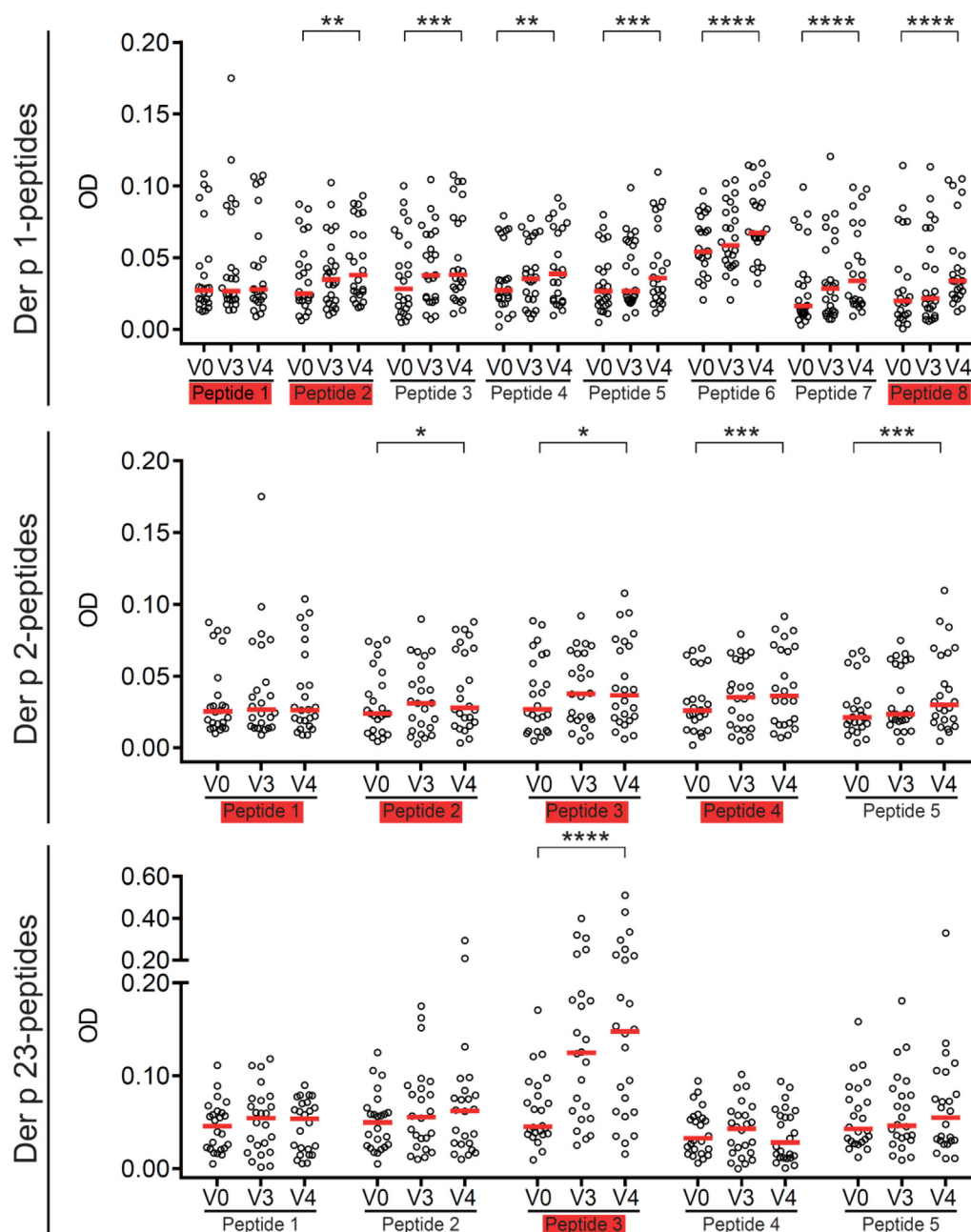


FIG 4. Development of IgG₄ responses to allergen-derived peptides in sera from patients in the HDMIT group during AIT. IgG₄ levels (y axes show OD levels) to Der p 1-, Der p 2-, and Der p 23-derived peptides (see Table E1) are displayed for different visits (x axes show values at V0 [baseline], V3 [33 weeks], and V4 [1 year]). Peptides that, on immunization of animals, have induced IgG antibodies blocking patients' IgE binding to an extent greater than 40%, as indicated in Table E1, are printed in red. Horizontal red bars indicate median values. Statistically significant differences are indicated as follows: * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$.

significant differences were found (Fig 6). None of the patients with IgE reactivity to Der p 5 or Der p 21 had a CSMS reduction greater than 50%. Likewise, no VAS score reduction greater than 50% was found for patients with sensitization to Der p 5 or Der p 21 (Fig 6). Similarly, there was no relevant VAS score reduction for the 2 patients (ie, patients 008 and 024) who were negative for Der p 1 and Der p 2, and no relevant reduction in CSMS was found for 1 of the patients with available data (ie, patient 024) (see Table E2).

Patients who were sensitized exclusively to Der p 1 and/or Der p 2 (Fig E6, A in this article's Online Repository at www.jacionline.org) but not to any of the other important HDM allergens (ie, Der p 5, Der p 7, Der p 21, and Der p 23) showed a greater reduction in symptoms after 1 year of treatment (a median VAS score reduction of 59.33% [$n = 10$] and median CSMS reduction of 38.24% [$n = 9$]) than did patients with additional sensitizations to Der p 5, Der p 7, Der p 21, and/or Der p 23 (a median VAS score reduction of 39.77% [$n = 14$] and median CSMS reduction of

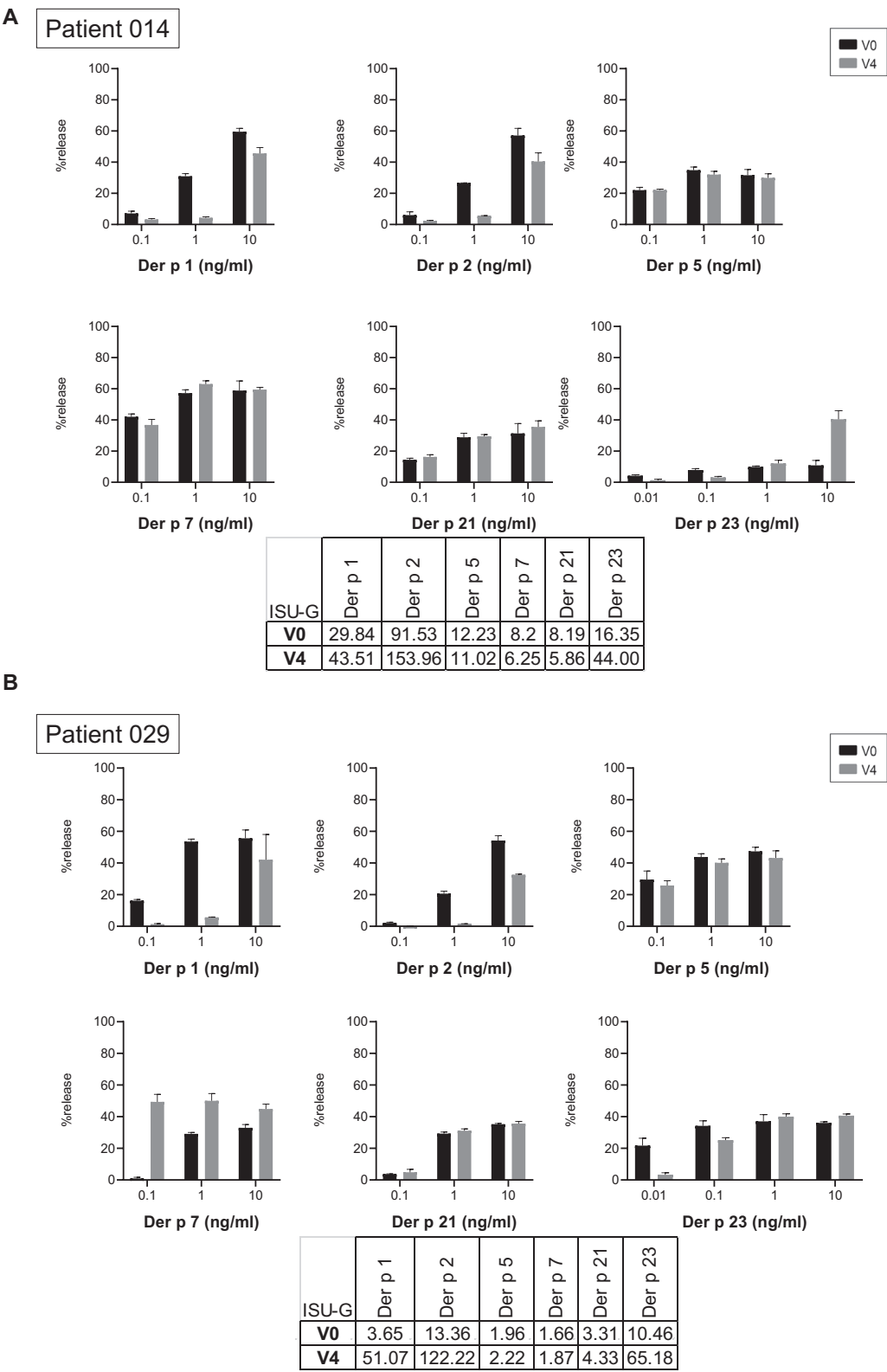


FIG 5. Effects of treatment-induced IgG antibodies on allergen-induced basophil activation. Basophils were loaded with baseline serum IgE from patient 014 (A) and patient 029 (B) and then exposed to increasing concentrations of HDM allergen molecules (x axes show concentrations of 0.1 ng/mL, 1 ng/mL, and 10 ng/mL) that had been preincubated with serum obtained before AIT (V0 [black bars]) or after 1 year of AIT (V4 [gray bars]). Percentages of total β -hexosaminidase release are shown on the y axes (means \pm SDs). Allergen-specific IgG levels measured at V0 and V4 are shown for each of the patients. ISU-G, ISAC standardized quality unit for IgG antibodies.

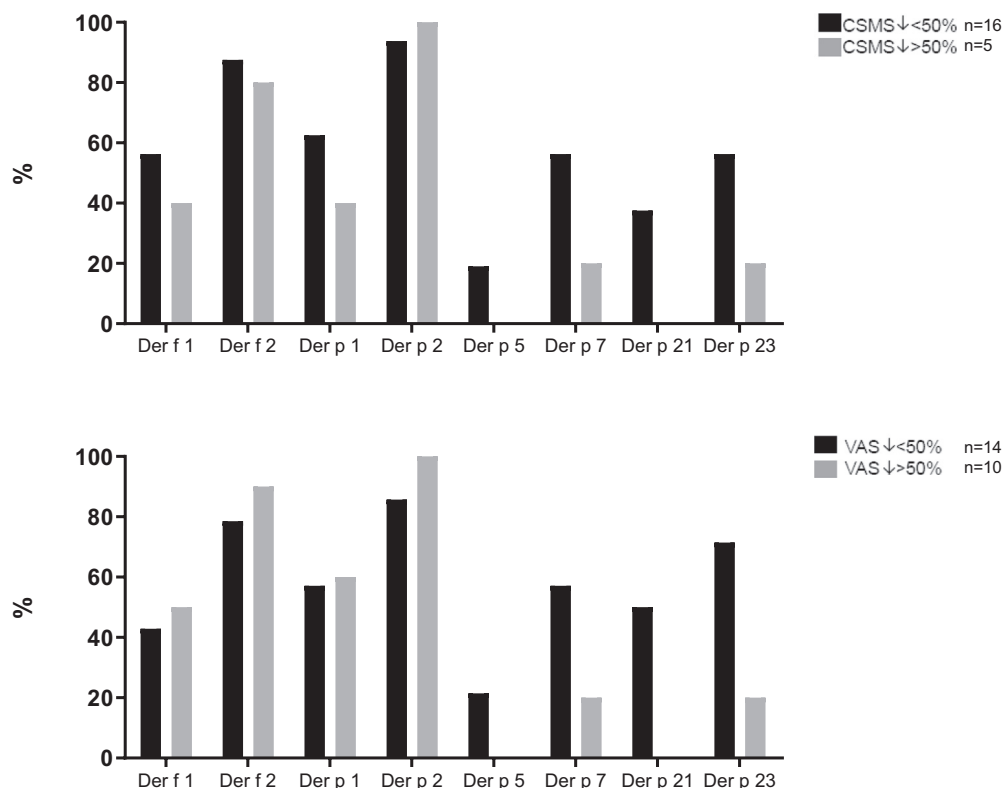


FIG 6. Percentages of patients with IgE reactivity (y axes) to the clinically relevant HDM allergen molecules (Der f 1, Der f 2, Der p 1, Der p 2, Der p 5, Der p 7, Der p 21, and Der p 23) (x axes) in the group with more (gray bars) or less (black bars) than a 50% reduction in CSMS and VAS scores between V0 (baseline) and V4 (1 year). The percentages of reduction were calculated as $\frac{(100 \times (V4 - V0))}{V0}$ and are shown for each patient in Table E2. Numbers of patients analyzed are indicated in the figure. Two patients without IgE sensitization to allergen molecules (patients 008 and 024) were not included.

17.89% [n = 12]) (Fig E6, B). However, these differences were also not significant. The VAS score and CSMS alterations of patients from the HDMA group who had not received AIT were different from the those of patients in the HDMIT group, with no advantage for patients sensitized only to Der p 1 and/or Der p 2 (see Table E3).

DISCUSSION

This is the first real-life study indicating that it may be possible to enhance the outcome HDM AIT by using a personalized approach based on the selection of patients according to their molecular sensitization profiles and by molecular monitoring of the development of AIT-induced allergen-specific IgG responses. We found that AIT with Alutard-SQ 510 induced allergen-specific IgG responses mainly against Der p 1 and Der p 2 and, to lower extent, to Der p 23 whereas no IgG responses to the other 3 important HDM allergens, Der p 5, Der p 7, and Der p 21, were detectable even after 1 year of treatment. The manufacturer of Alutard-SQ 510 does not provide information regarding the contents of the individual HDM allergens in the vaccine. However, Casset et al have shown that HDM SPT extracts from the company that produced Alutard-SQ 510 contained Der p 1 and/or Der p 2 and little Der p 7 but lacked Der p 5 and Der p 21.¹⁵ Similar findings were made for natural HDM extracts produced by other companies,¹⁵ and the analysis of an HDM AIT study also showed that there was no induction of IgG antibodies against

Der p 5, Der p 7, and Der p 21.²⁸ It is thus conceivable that natural HDM allergen extracts contain much less Der p 5, Der p 7, Der p 21, and Der p 23 than Der p 1 and Der p 2.

The SCIT-induced allergen-specific IgG response consisted of a specific IgG₁ and IgG₂ response that was maintained during treatment and a continuously rising IgG₄ response similar to that observed for pollen AIT and AIT with the recombinant grass pollen allergy vaccine BM32.⁴⁶ Support for a beneficial role of the SCIT-induced IgG response comes from our finding that the specific IgG that developed during SCIT reduced allergen-specific basophil activation. Because our results were obtained with basophils, which do not express inhibitory Fcγ receptors, the mechanism behind the reduction of allergen-specific basophil activation is most likely competition of IgG with IgE for allergen binding. Furthermore, suppression of allergen-induced basophil activation was associated with a reduction of allergen-specific IgE binding during testing with low amounts of microarrayed allergens, an *in vitro* assay that has been described to visualize the competition of blocking IgG antibodies with specific IgE.^{47,48} According to recent studies, it is also possible that SCIT-induced IgG antibodies may reduce effector cell activation by crosslinking the allergen/IgE/IgE receptor complex with the IgG-inhibitory receptor FcγRIIb,⁴⁹⁻⁵¹ although this mechanism was found to not be of relevance for Alutard-based AIT vaccines.⁵²

Previous studies have shown that adult patients with allergy do not develop new IgE sensitizations to respiratory allergens in the natural course of disease.^{13,53} Interestingly, we found that SCIT

with Alutard-SQ 510 also induced a *de novo* development of allergen-specific IgE in patients who were not sensitized against these allergens. We found that these *de novo*-induced allergen-specific IgE antibodies could lead to specific basophil activation when loaded onto basophils, but we have no evidence that they may be harmful considering that at the same time much higher levels of allergen-specific IgG antibodies appeared in the blood.

However, the most important finding from our real-life study was that SCIT with Alutard-SQ 510 induced only a partially protective IgG antibody response. SCIT-induced IgG responses were directed mainly against Der p 1 and Der p 2, and to a lesser extent to Der p 23, but not to the other 3 important HDM allergens, Der p 5, Der p 7, and Der p 21. Der p 5, Der p 7, Der p 21, and Der p 23, but not Der p 4, must be considered clinically relevant allergens for our patients because they induced strong basophil degranulation even when specific IgE levels were low. In this context, it should be noted that several other factors besides specific IgE levels such as structural allergen determinants (eg, number and proximity of IgE epitopes), as well as avidities of IgE antibodies, are important for the allergenic activity of an allergen.^{54,55} We even noted that not all of the Der p 1- and Der p 2-specific IgG antibodies induced by SCIT were directed against the major IgE binding sites of the allergens. Similar findings were recently made for patients who had received SCIT with a grass pollen allergy vaccine and a new recombinant grass pollen vaccine, BM32, which is based on carrier-bound peptides derived from the major IgE binding sites of the allergens.^{56,57} The comparison of IgG responses obtained by using the extract-based vaccine with the responses induced by BM32 showed that BM32 focused IgG responses against the IgE binding sites whereas the allergen extract-based vaccine also induced IgG against less relevant epitopes.⁵⁷

Alutard-SQ 510 induced protective IgG mainly to Der p 1 and/or Der p 2 and, to some extent, to Der p 23. The comparison of clinical outcomes according to CSMS and VAS score assessment indeed indicated that patients who are sensitized only to Der p 1 and/or Der p 2 benefited more from this treatment than patients who were also sensitized to other clinically relevant HDM allergens such as Der p 5, Der p 7, Der p 21, and Der p 23, which are not cross-reactive with Der p 1 and/or Der p 2.⁵⁸ Der p 4 was not considered a clinically relevant allergen because it did not induce relevant basophil degranulation in our patients. Other explanations for the differences in clinical outcomes are unlikely. First of all, the assessment of CSMS and VAS score was performed outside pollen seasons, and our patients did not have relevant sensitizations against other indoor allergens that played a role. Another possibility is that patients benefiting less from AIT had higher IgE levels against Der p 1 and/or Der p 2 and the therapy-induced IgG could block IgE binding in these patients less. However, this is not the case because after AIT basophil sensitivity decreased in all Der p 1- and Der p 2-sensitized patients approximately 10-fold regardless of Der p 1- and/or Der p 2-specific IgE levels. We therefore attribute the lower success rate of AIT in the patients sensitized to Der p 1 and/or Der p 2 and additional allergens to the lack of induction of protective IgG antibodies against the additional allergens.

It is a limitation of our study that only a limited number of patients could be studied. Nevertheless, the results obtained in this real-life study and those from an earlier HDM immunotherapy study²⁸ would suggest that one could increase the clinical

improvement in HDM-specific SCIT by using a personalized medicine approach that is either based on the stratification of patients with HDM allergy by molecular allergy diagnosis in patients who are mainly sensitized to Der p 1 and/or Der p 2 and/or by monitoring of SCIT by molecular measurement of IgG responses. It might therefore be possible to improve the success rate of HDM AIT with Alutard-SQ 510 by selecting for treatment those patients who show IgE reactivity mainly to Der p 1 and/or Der p 2 but not to Der p 5, Der p 7, and/or Der p 21. In addition, one may consider continuing treatment with other AIT vaccines if it turns out that the AIT vaccine that had been selected as the first option did not induce IgG antibodies against all the relevant allergens needed. Our results may be extendable to other populations with HDM allergy because they show IgE reactivity profiles that are similar to those of the patients in our study.⁵⁹⁻⁶¹ Furthermore, our results may also be applicable to other AIT vaccines because similar results were obtained for patients in a study of HDM AIT.²⁸ Of course, larger clinical studies with more patients with HDM allergy will be needed to confirm our initial results, but our study provides the first real-life evidence for such a personalized concept for HDM-specific AIT practice.

Clinical implications: Molecular diagnosis and molecular monitoring may improve the success of HDM-specific AIT in real life through a personalized approach.

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